α V β 6 Integrin Promotes Corneal Wound Healing

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PURPOSE. To appreciate the role of the integrin α V β 6 in corneal wound repair, corneal debridement and keratectomy in β 6 knockout (β 6^{-/-}) mice were examined.

METHODS. Either a 2-mm debridement or keratectomy was made in 129SVE wild type mice (WT) and β 6^{-/-} mice and allowed to heal for up to 4 months. The pattern of corneal restoration was studied "in vivo" by slit lamp and in tissue sections by means of both light and electron microscopy. In addition, α V β 6, α 6 β 4, laminin, and fibronectin were evaluated by indirect immunofluorescence microscopy and/or Western blot analysis.

RESULTS. α V β 6 expression was upregulated in migrating corneal epithelium after a keratectomy. Healing rates were unaffected in debridement wounds, but were significantly slowed in keratectomy wounds. Most dramatically, mice lacking α V β 6 had a severe defect in basement membrane zone (BMZ) regeneration. Levels of laminin were greatly reduced and no BMZ reformation was observed in transmission electron microscopy (TEM). In addition, hemidesmosome reformation was also impaired in the β 6^{-/-} mice. Analysis of the hemidesmosome component α 6 β 4 indicated that normal amounts of this integrin were synthesized, suggesting that the defect was in reassembly of the hemidesmosomes. Finally, fibronectin persisted in the BMZ for as long as 4 months after keratectomy in the β 6^{-/-} mice.

CONCLUSIONS. It is hypothesized that the lack of α V β 6 leads to reduced laminin production during wound repair. This lack of laminin prevents reassembly of the BMZ and mature hemidesmosomes after keratectomy in β 6^{-/-} mice. (*Invest Ophthalmol Vis Sci.* 2011;52:8505–8513) DOI:10.1167/iovs.11-8194

Integrins are a family of receptors that mediate cell-cell and cell-matrix adhesion,^{1–3} as well as contributing to a wide variety of cellular responses, including survival, proliferation, and migration.³ These heterodimeric transmembrane glycoproteins have both alpha (α) and beta (β) subunits. The combination of these 2 subunits results in at least 22 different cell surface receptors with wide ligand binding specificities.

Integrin α V β 6 is expressed at very low levels in epithelial cells of healthy adult mammals; however, its expression is

considerably upregulated in response to injury or inflammation.^{4,5} Absence of α V β 6 is linked to initiation and progression of periodontal disease,⁶ regulation of lung and skin inflammation,⁷ delay in epidermal wound healing,⁸ and suppression of pulmonary fibrosis.⁹ In the cornea, expression of α V β 6 is restricted to the epithelium. Under pathologic conditions, such as in patients with bullous keratopathy,¹⁰ and during wound healing, α V β 6 is upregulated, suggesting a role in corneal epithelial wound repair¹¹ and keratinocyte migration.¹² α V β 6 is a receptor for many of the components of the basement membrane zone (BMZ) including fibronectin, vitronectin, tenascin, and E-cadherins.^{13,14} It is also the receptor for the Arg-Gly-Asp (RGD) attachment site of both latency-associated peptide (LAP)-TGF- β 1 and LAP-TGF- β 3.^{9,15,16} Attachment of α V β 6 to latent TGF- β has been shown in several investigations to be involved in the activation of TGF- β (for review see references 17–20). Indeed, α V β 6-null mice recapitulate much of the phenotype of TGF- β 1-null mice.²¹

Because α V β 6 is known to be expressed in remodeling rat basal corneal epithelium and human bullous keratopathy,^{10,11} and is an activator of latent TGF- β ,^{9,15,16} we hypothesized that α V β 6 plays an important role in corneal repair when the BMZ is compromised by surgery. We addressed this hypothesis by using adult β 6 integrin-deficient mice (β 6^{-/-}) and comparing them with wild type (WT) mice.

MATERIALS AND METHODS

All studies were conducted in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. Adult male 129SVE WT (Taconic; Albany, NY) and 12- to 18-week-old male and female 129SVE β 6^{-/-} mice (a kind gift from Jack Lawler, BIDMC/Harvard Medical School, Boston, MA) were anesthetized and either a 2-mm superficial keratectomy or debridement was performed, as previously described.^{22,23} In total, 200 mice per strain were examined—96 mice were used for Western blot analysis, 8 for transmission electron microscopy (TEM), 36 for light microscopy, 36 for immunofluorescence, and 24 for whole mounts.

In Vivo Wound Healing Experiments

Either a 2-mm superficial keratectomy was created,²³ where the epithelium, BMZ, and anterior stroma were removed, or a 2-mm debridement was created,²² where only the epithelium was removed. Corneas were allowed to heal for up to 4 months. At the designated time, animals were euthanized and tissue was harvested and processed for indirect immunofluorescence microscopy, light microscopy, or Western blot analysis.

Determination of Wound Healing Rates

The healing process was monitored every day for the first week and then once every week under a slit lamp (Topcon Medical Systems, Inc.; Oakland, NJ). The corneas were photographed before and after 2% fluorescein stain, and the rate of epithelial healing was evaluated by measuring the wound size with image processing software (ImageJ v.1.5;

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developed by Wayne Rasband, National Institutes of Health; Bethesda, MD; <http://rsb.info.nih.gov/ij>). The data were averaged and analyzed for significant variations (** $P < 0.01$ and *** $P < 0.001$). Two-way ANOVA and Bonferroni post test were applied to compare means by row, and the fold enhancement was plotted (Prism 5.0; GraphPad Software, La Jolla, CA).

Indirect Immunofluorescence Microscopy

For frozen sections, the eyes were enucleated, frozen in OCT, 6- μ m sections were cut, and indirect immunofluorescence (IF) was performed.¹¹ For whole mount, the corneas were enucleated, fixed in prechilled 100% methanol and dimethyl sulfoxide (4:1) for 2 hours at 20°C and then stored in 100% methanol at 20°C until ready to use. The corneas were prepared for immunofluorescence as described in Pal-Ghosh et al.²⁴ The following primary antibodies were used on both the sections and whole mounts and incubated at 4°C overnight: α v β 6 (clone ch6.2A1: 2.36 μ g/mL in TTBS + 0.1% BSA)²⁵; β 4 (clone 346-11A: 5 μ g/mL in TTBS/5% donkey serum: BD Pharmingen; San Diego, CA); laminin (0.76 μ g/mL in 10% donkey serum in PBS: Dako; Carpinteria, CA); and fibronectin (C-20: 2 μ g/mL in TTBS/5% donkey serum: Santa Cruz Biotechnology; Santa Cruz, CA). The following secondary antibodies from Jackson ImmunoResearch (West Grove, PA) conjugated to either fluorescein or rhodamine were then used with the same blocking conditions as the primary antibody associated with it and incubated at either room temperature for 1 hour (sections) or 4°C overnight (whole mount): donkey anti-human IgG (α v β 6); donkey anti-rat IgG (β 4); donkey anti-rabbit

IgG (laminin); and donkey anti-goat IgG (fibronectin). Sections and whole mounts were mounted and coverslipped with mounting media containing DAPI (Vectashield; Vector Laboratories, Burlingame, CA), a marker of all cell nuclei. The sections were examined under both a fluorescence microscope (Nikon Eclipse E800; Nikon, Melville, NY) equipped with a digital camera (SPOT; Diagnostic Instruments, Sterling Heights, MI) and with a confocal microscope (TCS-SP5; Leica Microsystems, Bannockburn, IL). Negative controls where the primary antibody was omitted were run with all experiments. As an additional control, irrelevant antibodies of the same isotype were compared to ensure specificity. At least 3 corneas per condition per antibody were observed.

The images captured on the confocal microscope were further imaged with image analysis software (Image Pro Plus v.7; Media Cybernetics, Bethesda, MD). The confocal file was uploaded and an orthogonal image was created.

Light Microscopy

Corneas were enucleated and either fixed in 1/2 strength Karnovsky's for transmission electron microscopy (TEM) or in 4% paraformaldehyde for hematoxylin-eosin (H&E). For TEM, corneas were processed as previously published.²⁶ Tissues were cut on an ultramicrotome (LKB; Bromma, Sweden) at 60–90 Å thick and examined with an electron microscope (Philips 410; Philips Electronics N.V., Eindhoven, The Netherlands). For H&E, 6- μ m sections were cut and stained. The sections were examined under a light microscope

FIGURE 1. Immunolocalization of α v β 6 (green) in WT mouse corneas: (A) unwounded, (B) 24 hours, (C) 48 hours, (D) 4 days, and (E) 4 weeks after keratectomy. (C, *inset*) WT mouse cornea 48 hours after debridement. In (F) and (G) whole mount corneas were examined by confocal microscopy 48 hours after keratectomy. (G) A 3-D software reconstruction (Image Pro Plus v.7; Media Cybernetics). Note the abrupt increase of expression in the basal cells migrating to cover the wound area. Blue in (G), DAPI, a marker of cell nuclei. Arrows denote the original wound edge. Bar, 50 μ m.

FIGURE 2. Western blot analysis of $\alpha\text{v}\beta 6$ in WT mouse corneas after a keratectomy wound. (A) Representative Western blot images documenting the increased expression of $\alpha\text{v}\beta 6$, and the corresponding stain (Ponceau S; Sigma) of the blot to document equal loading of samples (region from 40 to 70 kDa). Experiments were repeated three times using at least six mice per time point. (B) Density of the bands, quantitated and plotted.

(Nikon Eclipse E800; Nikon) equipped with a digital camera (Olympus DP70; Olympus America Inc.; Melville, NY).

Western Blot Analysis

Immediately after euthanizing the mice, the epithelium was scraped from limbus to limbus, flash frozen in liquid nitrogen, and processed for Western blot analysis, as previously described.²⁷ Eight corneas were used for each experimental condition. Tissues were homogenized and lysed, and the protein was purified and assayed (Biorad; Hercules, CA). Equal amounts of total protein were loaded on a nonreducing 4%–20% Tris-Glycine gel (Invitrogen, Carlsbad, CA). Proteins were transferred to a membrane (Immobilon-P; Millipore, Billerica, MA), which was then stained (Ponceau S; Sigma) to check for transfer efficiency. Membranes were blocked with 5% milk in TBS and then probed, as previously described²⁷ with the following primary antibodies and incubated at 4°C overnight: $\alpha\text{v}\beta 6$, clone ch6.2A1 (0.5–1 $\mu\text{g}/\text{mL}$); $\beta 4$ (1:500)²⁸; and laminin antibody (0.76 $\mu\text{g}/\text{mL}$). The appropriate HRP-conjugated secondary antibody (Jackson ImmunoResearch) was then incubated with the membranes for 1 hour at room temperature: donkey anti-human IgG ($\alpha\text{v}\beta 6$); donkey anti-rabbit IgG ($\beta 4$); and donkey anti-rabbit IgG (laminin). Immunoreactive bands were visualized (Biospectrum AC imaging system; UVP, LLC, Upland, CA) using enhanced chemiluminescence Western blot detection reagents (Millipore). Experiments were repeated two to three times. Software was used to measure intensity of the bands (ImageJ v.1.5) and plot the fold enhancement for the values (Prism 5.0; GraphPad Software).

RESULTS

Healing of Keratectomy Wounds Is Accompanied by Enhanced Expression of $\alpha\text{v}\beta 6$ Integrin in the Basal Epithelial Cells

We have previously demonstrated that $\alpha\text{v}\beta 6$ is upregulated in response to keratectomy and debridement wounds in rat cornea.¹¹ To confirm our rat experiments, we examined the spatial and temporal changes of $\alpha\text{v}\beta 6$ in response to wounds in 129SVE WT mice. In unwounded corneas (Fig. 1A), little, if any $\alpha\text{v}\beta 6$ was present; however, 24 hours after keratectomy (Fig. 1B), the number of cells with $\alpha\text{v}\beta 6$ increased, localizing in the basal epithelial cells that were migrating to cover the wound area. $\alpha\text{v}\beta 6$ continued to be present within this area at 48 hours (Figs. 1C, 1F, 1G), through 4 days (Fig. 1D) and 1 week (data not shown). It then gradually decreased and returned to baseline by 4 weeks (Fig. 1E). No upregulation of $\alpha\text{v}\beta 6$ was observed in the peripheral epithelial cells, as can be seen in Figures 1B, 1C, 1F, and 1G. In contrast to rats, $\alpha\text{v}\beta 6$ was not enhanced in the migrating epithelium in debridement wounds in 129SVE mice (Fig. 1C, inset).

To confirm the apparent increase in $\alpha\text{v}\beta 6$ in the epithelial cells within the wound area after keratectomy, tissue was collected and processed for Western blot analysis. The data from the Western blot analysis agreed with that observed in immunofluorescence (Fig. 2). Low levels of $\alpha\text{v}\beta 6$ were observed in the epithelium of unwounded (UnW) control corneas, and increased at 1, 2, and 4 days (D), and 1 week (w) post-keratectomy. The protein amount then began to decrease by 2 weeks. Stained (Ponceau S; Sigma) membrane showed equal loading (Fig. 2A).

$\alpha\text{v}\beta 6$ Integrin Plays a Main Role in Epithelial Cell Migration after Keratectomy

Because $\alpha\text{v}\beta 6$ was observed in the migrating basal corneal epithelial cells in WT mice, we decided to examine the wound-healing rate in WT and $\beta 6^{-/-}$ mice in vivo to see if $\alpha\text{v}\beta 6$ played a role in wound healing. As can be seen in Figure 3 (red and blue lines), 24 hours after a keratectomy, most of the corneas had recovered 25% of their surface, without differences among mouse genotypes. However, an increase in the migration rate in the WT mice was observed after 24 hours. The rate of migration was significantly delayed in the $\beta 6^{-/-}$ corneas, especially at 32 and 40 hours ($P < 0.001$) (Fig. 3).

FIGURE 3. Graphical documentation of healing rates of WT and $\beta 6^{-/-}$ mice after 2-mm debridement or keratectomy. At least six mice were used per time point. ** $P < 0.01$, *** $P < 0.001$.

To compare the effect of $\alpha\text{v}\beta 6$ on wounds with or without damage to the BMZ, we also examined the wound healing rate of WT and $\beta 6^{-/-}$ mice with debridement wounds. As with the keratectomy, the epithelial wound healing rates were approximately the same for both strains of mice up until 16 hours post wounding (Fig. 3, green and black lines). However, at 16 hours post debridement, whether it was WT or $\beta 6^{-/-}$ mice, there was an increase in the wound healing rate. This increase occurred approximately 8 hours earlier than in the corneas with a keratectomy (Fig. 3, red and blue lines). By 24 hours post debridement, both strains of mice had resurfaced $> 50\%$ of the original wound size. No statistically significant difference was observed after debridement between WT and $\beta 6^{-/-}$ mice at any time point (Fig. 3).

$\alpha\text{v}\beta 6$ Integrin Mediates Attachment of Epithelium to Extracellular Matrix (ECM) during Wound Healing after Keratectomy

Once the defect is closed and epithelial migration is over, new cell-cell and cell-ECM adhesions need to be created for both restoration of barrier function and anchoring of the epithelium.

Assembly of hemidesmosomes in the new epithelial basal layer is essential to maintain the integrity of the epithelia.²⁹ In both the WT and $\beta 6^{-/-}$ mice, the unwounded corneas look similar (Figs. 4A, 4B). By 4 days post keratectomy, the epithelium in both strains covered the wound area (Figs. 4C, 4D). In WT mice, the epithelium appeared to be forming adhesions to the stroma (Fig. 4C), whereas in $\beta 6^{-/-}$ mice, the epithelium had a large area that was not attached to the stroma (Fig. 4D). By 2 weeks post keratectomy, the WT mice appeared to have returned to normal and more adhesions appeared to have been formed (Fig. 4E). However, in the $\beta 6^{-/-}$ mice, there was still a large area where the epithelium was not attached to the stroma (Fig. 4F). This detachment was observed in six of six of the $\beta 6^{-/-}$ mice and in zero of six of the WT mice. By 4 weeks, the WT mouse corneas had returned to that seen in unwounded (Fig. 4G); whereas the $\beta 6^{-/-}$ mouse cornea's morphology (Fig. 4H) was more similar to that seen in the WT mice at 2 weeks (Fig. 4E). On slit lamp examination, the epithelium could be seen to be loosely adherent in the $\beta 6^{-/-}$ mice with blister-like structures (Fig. 4I). These blister-like structures were not present in the WT mice (data not shown).

FIGURE 4. Morphologic analysis of WT (A, C, E, G) and $\beta 6^{-/-}$ (B, D, F, H) in (A, B) unwounded corneas, (C, D) 4 days, (E, F) 2 weeks, and (G, H) 4 weeks post keratectomy. Note that epithelium is loosely adherent in $\beta 6^{-/-}$ after wounding. Bar, 100 μm . (I) Slit lamp image (magnification, $\times 40$) of $\beta 6^{-/-}$ cornea 1 week after keratectomy. Note the blister-like structure extends across the cornea (arrows).

α V β 6 Integrin Helps to Restore the BMZ after Keratectomy by Upregulating New Laminin and Promoting Assembly of Hemidesmosomes

Because the wound-healing rate was slowed and the adherence of the epithelium to the stroma was defective in β 6 $^{-/-}$ mice after a keratectomy wound, we examined the structure of the BMZ by TEM. One month after debridement, in both strains of mice (Figs. 5A, 5B), the BMZ looked similar to that seen in unwounded corneas (data not shown). One month after keratectomy, the WT BMZ appeared to be reforming hemidesmosomes and a nearly continuous BMZ was observed (Fig. 5C). In contrast, the number of mature hemidesmosomes was greatly reduced in the β 6 $^{-/-}$ mouse corneas and there

were no signs of BMZ reformation (Fig. 5D). By 4 months post keratectomy, the BMZ of the WT mice (Fig. 5E) looked similar to that seen in unwounded corneas; however, the amount of hemidesmosomes present in β 6 $^{-/-}$ mice was markedly reduced compared with WT, and only a faint suggestion of BMZ was observed (Fig. 5F).

This apparent lack of BMZ and reduced number of hemidesmosomes in the β 6 $^{-/-}$ mice after a keratectomy leads us to question, how is the lack of α V β 6 disrupting their reformation? To examine this question, two markers were used. A laminin antibody generated using rat yolk sac tumor laminin as an antigen, which localizes to the BMZ, but does not identify a specific laminin molecule, and α 6 β 4, which localizes within

FIGURE 5. Transmission electron micrographs of WT (A, C, E) and β 6 $^{-/-}$ (B, D, F) mouse corneas 1 month after debridement (A, B), and 1 (C, D) and 4 months (E, F) post keratectomy. Note the complete absence of BMZ and the greatly reduced number of electron dense hemidesmosomes 1 month after keratectomy in the β 6 $^{-/-}$ mice. Also note the epithelial projections into the stroma that may help anchor the epithelium. Arrows indicate location of BMZ. Bar, 500 nm.

hemidesmosomes at the BMZ (Fig. 6). As with humans, rats, and chickens,^{11,30–32} an uninterrupted linear pattern of laminin was observed beneath the epithelium in unwounded corneas of WT and $\beta 6^{-/-}$ mice (Figs. 6A, 6E). Laminin colocalized with $\alpha 6\beta 4$ in both genotypes before wounding (Figs. 6A1, 6E1). After keratectomy, the BMZ was removed and therefore, there was no laminin or $\alpha 6\beta 4$ localization within the wound area in either strain of mice (data not shown). In WT mice 4 days post keratectomy, laminin (Fig. 6B) and $\alpha 6\beta 4$ (Fig. 6B1) colocalized and appeared just below the epithelium; however, they were not yet continuous, but rather, patchy and thickened. By 1 week, the patchiness of both laminin and $\alpha 6\beta 4$ began to disappear (Figs. 6C, 6C1). By 4 weeks, both markers continued to colocalize and the thickening was disappearing (data not shown) and by 2 months (Figs. 6D, 6D1), it was

similar to unwounded (Figs. 6A, 6A1). On the other hand, 4 days after a keratectomy in $\beta 6^{-/-}$ mice (Figs. 6F, 6F1), small patches of laminin and $\alpha 6\beta 4$ were present, but the majority of the wound area had little staining. One week post keratectomy (Figs. 6G, 6G1), there appeared to be more patches of staining. This increase in patchy localization continued at 2 (Figs. 6H, 6H1) and 4 weeks (data not shown). By 4 months, laminin (Fig. 6I) and $\alpha 6\beta 4$ (Fig. 6I1) colocalized where the epithelium and the stroma met; however, in the area where the epithelium did not appear to be adhering to the stroma, laminin was not present.

To confirm the amounts of laminin and $\alpha 6\beta 4$ in the migrating epithelium over time, we performed Western blot analysis. In Figure 7.1, it can be seen that laminin levels increased rapidly in WT mice after wounding and continued to rise for at least 1 week

FIGURE 6. Immunolocalization of laminin (A–I, red) and $\alpha 6\beta 4$ (A1–I1, green) in WT (A–D, A1–D1) and $\beta 6^{-/-}$ (E–I, E1–I1) mice. Proteins were localized in unwounded corneas (A, A1, E, E1) and 4 days (B, B1, F, F1), 1 week (C, C1, G, G1), 2 weeks (H, H1), 2 months (D, D1) and 4 months (I, I1) post keratectomy. Note that laminin and $\alpha 6\beta 4$ were analyzed on single sections that are shown separately for ease of viewing. DAPI, a marker of all cell nuclei, blue. Bar, 50 μ m. At least four corneas per time point were analyzed.

FIGURE 7. Representative Western blot images documenting the increased expression of laminin (1A) and α 6 β 4 (2A) after keratectomy wounds. Stain (Ponceau S; Sigma) of the blots was used to document equal loading of samples (data not shown). Experiments were repeated two or three times using at least six mice per time point. The density of the bands was quantitated and fold enhancement was plotted (1B, 2B). Note: Only epithelium was included in these samples.

in a representative experiment. In contrast, only very low levels of laminin were present in the epithelium of β 6 $^{-/-}$ mice after a keratectomy wound. As seen in Figure 7.2, the amount of α 6 β 4 increased in both strains after keratectomy, with WT peaking by 2 days post keratectomy and β 6 $^{-/-}$ by 4 days. The levels of α 6 β 4 then decreased to unwounded by 2 weeks in both strains.

Fibronectin Persists after Keratectomy in α V β 6 Null Mice

Release of fibronectin within the injured area is critical for healing. The released fibronectin acts as a temporary matrix allowing attachment of the new migrating cells.⁴ Fibronectin is also known to be a ligand for α V β 6.³³ We, therefore, examined

the spatial and temporal changes of fibronectin in the WT and β 6 $^{-/-}$ mice after keratectomy (Fig. 8). In unwounded corneas of both strains of mice, no fibronectin was observed (data not shown). At 24 hours, fibronectin was observed from edge-to-edge in the wounded area beneath the migrating epithelium in both WT and β 6 $^{-/-}$ mice, and no fibronectin was observed in the peripheral tissue (data not shown). In both strains of mice, fibronectin peaked at 1 week (Figs. 8A, 8B). After 1 week, fibronectin decreased suddenly in WT mice, and only a little staining was observed at 2 weeks (data not shown). By 2 months, fibronectin rarely was observed in WT mice (Fig. 8C). In contrast, β 6 $^{-/-}$ mice continued to have fibronectin at 2 and 4 months (Figs. 8D and 8E, respectively).

FIGURE 8. Immunolocalization of fibronectin (red) in WT (A, C) and β 6 $^{-/-}$ (B, D, E) mice: (A, B) 1 week, (C, D) 2 and (E) 4 months post keratectomy. Note that no fibronectin was present in unwounded corneas, or beyond 2 weeks after wounding in WT cornea. DAPI, a marker of all cell nuclei, blue. Bar, 50 μ m.

DISCUSSION

Healthy corneal epithelium is essential for maintaining transparency, avoiding infection and maintaining corneal integrity. To preserve the structure, the epithelium anchors to the stroma ECM through the BMZ. We previously observed a spatial/time correlation between $\alpha\text{v}\beta 6$ upregulation in basal corneal epithelial cells and laminin expression in the BMZ of rats after a keratectomy wound.^{11,12} With the present study, we extended our work to demonstrate that $\beta 6^{-/-}$ mice did not restore the BMZ or reform mature hemidesmosomes after a keratectomy wound. By 1 month after keratectomy, WT mice showed a nearly fully restored BMZ by TEM, with restoration of both lamina densa and lucida, as well as a high density of hemidesmosomes. Dramatically, all these components were missing in the $\beta 6^{-/-}$ mice, indicating that $\alpha\text{v}\beta 6$ is required for regeneration of the BMZ and hemidesmosomes. In contrast, after debridement, both WT and $\beta 6^{-/-}$ mouse corneas showed a normal appearance in both BMZ and hemidesmosomes.

Immediately after photorefractive keratectomy, humans, rats, and chickens all deposit laminin underneath the epithelium.^{11,30-32} Concordant with these works, WT mouse corneas showed a continuous laminin line limbus-to-limbus by 2 weeks after keratectomy. However, within the damaged zone in the $\beta 6^{-/-}$ mice, laminin was absent, weeks and even months after a keratectomy, and when present, only present as clusters within the healing BMZ. Even by 4 months, continuous laminin localization was not observed, which is in agreement with the lack of BMZ observed by TEM. Western blot analysis and IF results demonstrated that laminin production decreased and its assembly into BMZ was impaired. One possible explanation for the lack of laminin production is that activation of TGF- $\beta 1$ or - $\beta 3$ may be impaired by the lack of $\alpha\text{v}\beta 6$. This possibility is supported by studies suggesting that laminin production is increased in epithelial cells in response to autocrine TGF- $\beta 1$ activation.^{34,35} We hypothesize that laminin expression may be upregulated in the corneal epithelium by $\alpha\text{v}\beta 6$ -activated TGF- β .³⁶ This type of activation has been reported in numerous systems,¹⁷⁻²⁰ and $\alpha\text{v}\beta 6$ expression has been frequently linked to fibrosis.³⁷⁻⁴¹

Both loose epithelium and lack of hemidesmosomes have been observed in mice lacking $\alpha 6\beta 4$ integrin.⁴² Our $\beta 6^{-/-}$ mouse corneas appear to have a greatly reduced number of mature hemidesmosomes after 1 and 4 months post keratectomy. An evident colocalization of $\alpha 6\beta 4$ and laminin is observed in both WT and $\beta 6^{-/-}$ unwounded corneas. This colocalization is also observed in healing corneas of WT mice after keratectomy; therefore, this strongly suggests that $\alpha 6\beta 4$ is a receptor for laminin during BMZ repair, and that the presence of laminin is necessary to assemble $\alpha 6\beta 4$ into hemidesmosomes, during corneal epithelial BMZ repair.

In addition to the defective restoration of the BMZ, we observed that wound closure in the $\beta 6^{-/-}$ mice was slowed after a keratectomy. Although some studies have suggested that lack of $\alpha\text{v}\beta 6$ compromises epidermal repair,^{6,8} others have proposed that it does not,^{7,21} or only the re-epithelialization is compromised in $\beta 6^{-/-}$ diabetic mice, but not in $\beta 6^{-/-}$ healthy mice.⁴³ In the current work, we observed significant differences in the rate of migration between WT and $\beta 6^{-/-}$ mice when the BMZ was removed by keratectomy. Interestingly, $\beta 6^{-/-}$ mice did not show any significant slowing of migration after a debridement wound, nor did $\alpha\text{v}\beta 6$ appear to be enhanced after a debridement wound. These data suggest that $\alpha\text{v}\beta 6$ plays a major role in migration only when the BMZ is damaged. The data also suggest that matrix might be responsible for inducing $\alpha\text{v}\beta 6$.

Because fibronectin is observed in the wound-healing stroma after a keratectomy, and is believed to be involved in

epithelial migration⁴ and is known to be a substrate for $\alpha\text{v}\beta 6$,³³ there may be an interaction between the $\alpha\text{v}\beta 6$ in the migrating epithelium and the upregulated fibronectin in the stroma that increases the speed of epithelial migration over the wound area after a keratectomy. In the healing cornea, fibronectin is released, facilitating both epithelial migration and attachment to ECM.^{44,45} When the wound defect is closed, fibronectin is lost, correlating with the appearance of laminin. As in a previous experiment in rabbits,⁴⁶ loss of fibronectin expression correlating with BMZ restoration was evident in the WT mice; however, the loss of fibronectin and appearance of laminin was not observed in the $\beta 6^{-/-}$ mice. Instead, interrupted costaining of fibronectin and laminin remained in the later time points, suggesting that lack of BMZ restoration was associated with the continuing expression of fibronectin.

With the current work, we demonstrate that $\alpha\text{v}\beta 6$ is upregulated in the epithelium during corneal wound healing when the BMZ is damaged in WT mice. The upregulation of $\alpha\text{v}\beta 6$ promotes epithelial migration in wounds where the BMZ is removed, and leads to upregulation of laminin. In addition, in mice lacking $\alpha\text{v}\beta 6$, hemidesmosome restoration is compromised after keratectomy wounds and this correlates with the absence of laminin in the BMZ.

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